Malignant astrocytomas of elderly patients lack favorable molecular markers: an analysis of the NOA-08 study collective

Wiestler, B ; Claus, R ; Hartlieb, S A ; Schliesser, M G ; Weiss, E K ; Hielscher, T ; Platten, M ; Dittmann, L M ; Meisner, C ; Felsberg, J ; Happold, C ; Simon, M ; Nikkhah, G ; Papsdorf, K ; Steinbach, J P ; Sabel, M ; Grimm, C ; Weichenhan, D ; Tews, B ; Reifenberger, G ; Capper, D ; Müller, W ; Plass, C ; Weller, M ; Wick, W

Abstract: Background The number of patients age >65 years with malignant gliomas is increasing. Prognosis of these patients is worse compared with younger patients. To determine biological differences among malignant gliomas of different age groups and help to explain the survival heterogeneity seen in the NOA-08 trial, the prevalence and impact of recently established biomarkers for outcome in younger patients were characterized in elderly patients. Methods Prevalences of mutations of isocitrate dehydrogenase 1 (IDH1) and histone H3.3 (H3F3A), the glioma cytosine-phosphate-guanine island methylator phenotype (G-CIMP), and methylation of alkylpurine DNA N-glycosylase (APNG) and peroxiredoxin 1 (PRDX1) promoters were determined in a representative biomarker subset (n = 126 patients with anaplastic astrocytoma or glioblastoma) from the NOA-08 trial. Results IDH1 mutations (R132H) were detected in only 3/126 patients, precluding determination of an association between IDH mutation and outcome. These 3 patients also displayed the G-CIMP phenotype. None of the IDH1 wild-type tumors were G-CIMP positive. Mutations in H3F3A were absent in all 103 patients sequenced for H3F3A. MassARRAY analysis of the APNG promoter revealed generally low methylation levels and failed to confirm any predictive properties for benefit from alkylating chemotherapy. Neither did PRDX1 promoter methylation show differential methylation or association with outcome in this cohort. In a 170-patient cohort from The Cancer Genome Atlas database matched for relevant prognostic factors, age 65 years was strongly associated with shorter survival. Conclusions Despite an age-independent stable frequency of O(6)-methylguanine-DNA methyltransferase (MGMT) promoter hypermethylation, tumors in this age group largely lack prognostically favorable markers established in younger glioblastoma patients, which likely contributes to the overall worse prognosis of elderly patients. However, the survival differences hint at fundamental further differences among malignant gliomas of different age groups.

DOI: https://doi.org/10.1093/neuonc/not043

W (2013). Malignant astrocytomas of elderly patients lack favorable molecular markers: an analysis of the NOA-08 study collective. Neuro-Oncology, 15(8):1017-1026.
DOI: https://doi.org/10.1093/neuonc/not043

Malignant astrocytomas of elderly patients lack favorable molecular markers: An analysis of the NOA-08 study collective

Benedikt Wiestler, Rainer Claus, Sabine A. Hartlieb, Maximilian G. Schliesser, Elisa K. Weiss, Thomas Hielscher, Michael Platten, Laura M. Dittmann, Christoph Meisner, Jörg Felsberg, Caroline Happold, Matthias Simon, Guido Nikkhah, Kirsten Papsdorf, Joachim P. Steinbach, Michael Sabel, Christiane Grimm, Dieter Weichenhan, Björn Tews, Guido Reifenberger, David Capper, Wolf Müller, Christoph Plass, Michael Weller and Wolfgang Wick for the Neurooncology Working Group (NOA) of the German Cancer Society

German Cancer Consortium (DKTK), Heidelberg, Germany (B.W., R.C., S.A.H., M.G.S., E.K.W., T.H., M.P., L.M.D., C.G., D.W., B.T., D.C., W.M., C.P., W.W.), Departments of Neurooncology (B.W., E.K.W., M.P., C.G., W.W.), National Centre for Tumour Diseases, and Neuropathology (D.C., W.M.), University of Heidelberg, Im Neuenheimer Feld 400, and Clinical Cooperation Units Neurooncology (B.W., S.A.H., M.G.S., E.K.W., C.G., W.W.) and Neuropathology (D.C., W.M.), Divisions of Epigenomics and Cancer Risk Factors (R.C., D.W., C.P.), Biostatistics (T.H.) and Division of Molecular Genetics (L.M.D.), German Cancer Research Centre, D-69120 Heidelberg; Department of Medical Biometry (C.M.), Westbahnhofstraße 55, D-72070 Tübingen, University of Tübingen, Hoppe-Seyler-Strasse 3, D-72076 Tübingen; Departments of Neuropathology (J.F., G.R.) and Neurosurgery (M.Sa.), Heinrich Heine University, Moorenstrasse 5, D-40225 Düsseldorf; Department of Neurosurgery (M.Si.), University of Bonn, Sigmund-Freud-Strasse 25, D-53105 Bonn; Department of Stereotactic Neurosurgery (G.N.), University Clinic Freiburg, Breisacher Strasse 64, D-79106 Freiburg; Department of Radiation Oncology (K.P.), University Hospital Leipzig, Stephanstrasse 9a, D-04103 Leipzig; Dr. Senckenbergisches Institute for Neurooncology (J.P.S.), University of Frankfurt, Schleusenweg 2-16, D-60528 Frankfurt; CHS Group Molecular Mechanism of Tumor Cell Invasion (B.T.), German Cancer Research Centre, D-69120 Heidelberg, all Germany; Department of Neurology (C.H., M.W.), University Hospital Zurich, Frauenklinikstrasse 26, CH-8091 Zurich, Switzerland

Current address: Dept. of Medicine, Div. Hematology/Oncology, University of Freiburg Medical Center, Hugstetterstr. 55, D-79106 Freiburg (R.C.)

Running Title: Molecular biomarkers of gliomas in the elderly

Corresponding author: Wolfgang Wick, MD

Corresponding author's address: Department of Neurooncology

Neurology Clinic & National Centre for Tumour Diseases

University of Heidelberg

Im Neuenheimer Feld 400

D-69120 Heidelberg, Germany

Corresponding author's phone and fax: Phone: +49 (0)6221/56-7075

Fax: +49 (0)6221/56-7554

Corresponding author's e-mail address: wolfgang.wick@med.uni-heidelberg.de

Funding: The current research was supported within the Brain Tumor Network (BTNplus; Subproject 6) of the National Genome Research Network (NGFNplus) by the Federal Ministry of Education and Research (BMBF), and the charitable Hertie Foundation.

Conflict of Interest: WW and MW received payments for lectures and advisory board participation from MSD, a company which manufactures temozolomide, Roche, Merck (MW) and Eli Lilly (WW). WW received funding for research from Apogenix, Boehringer Ingelheim, Eli Lilly and MSD. MW received funding for research from Merck, MSD and Roche.

ABSTRACT

Background: The number of patients > 65 years of age with malignant gliomas is increasing. Prognosis of these patients is worse compared to younger patients. To determine biological differences between malignant gliomas of different age groups and help to explain the survival heterogeneity seen in the NOA-08 trial, the prevalence and impact of recently established biomarkers for outcome in younger patients were characterized in elderly patients.

Methods: Prevalence of *isocitrate dehydrogenase 1* (*IDH1*) and *histone H3.3* (*H3F3A*) mutations, the glioma CpG island methylator phenotype (G-CIMP) and methylation of the *alkylpurine DNA N-glycosylase* (*APNG*) and *peroxiredoxin 1* (*PRDX1*) promoters was determined in a representative biomarker subset (n=126 patients with anaplastic astrocytoma or glioblastoma) from the NOA-08 trial.

Results: *IDH1* mutations (R132H) were detected in only 3/126 patients, precluding determination of an association between *IDH* mutation and outcome. These 3 patients also displayed the G-CIMP phenotype. None of the *IDH1* wildtype tumors were G-CIMP positive. Mutations in *H3F3A* were absent in all 103 patients sequenced for *H3F3A*. MassARRAY analysis of the *APNG* promoter revealed generally low methylation levels and failed to confirm any predictive properties for benefit from alkylating chemotherapy. Neither did *PRDX1* promoter methylation show differential methylation or association with outcome in this cohort. In a 170 patient cohort from the TCGA database matched for relevant prognostic factors, age ≥ 65 years was strongly associated with shorter survival.

Conclusions: Despite an age-independent stable frequency of *O6-methyl-guanyl-methyl-transferase (MGMT)* promoter hypermethylation, tumors in this age group largely lack prognostically favorable markers established in younger glioblastoma patients, which likely contributes to the overall worse prognosis of elderly patients. However, the survival differences hint at fundamental further differences between malignant gliomas of different age groups.

KEY WORDS

Glioblastoma, *IDH* mutation, *H3F3A* mutation, G-CIMP, *APNG* methylation, *PRDX1* methylation

INTRODUCTION

Glioblastoma (WHO grade IV) is the most common intrinsic brain tumor. The prognosis for patients suffering from this disease remains dismal. Age in particular is a strong negative predictor for survival, resulting in a population-based median survival of elderly patients (i.e., older than 65 years) of less than 6 months [1]. As glioblastoma incidence is strongly increasing with age, soon more than half of all glioblastoma patients will be considered elderly [2]. Improving the therapy of these patients is therefore one of the major challenges in neurooncology [3].

The standard of care in elderly patients is currently ill-defined. This is in part due to the exclusion of elderly patients in many clinical trials. The EORTC-NCIC trial, which defined concomitant and adjuvant radiochemotherapy with temozolomide as the standard of care, excluded patients older than 70 years [4]. In elderly patients receiving combined radiochemotherapy, treatment-associated toxicity seems to be higher compared to younger patients [5]. This especially holds true for radiation-related neurotoxicity, which demonstrates a clear age-dependency [6,7]. To date, involved-field radiotherapy alone is recommended as the standard first-line therapy after biopsy or resection in elderly patients [8]. However, due to the risk of radiation-induced neurotoxicity, temozolomide alone has been explored as a treatment option. Several studies have reported a median overall survival comparable to radiotherapy alone with only modest toxicity of temozolomide in this population [9,10]. To directly compare radiotherapy alone versus temozolomide alone, the German Neuro-Oncology Working Group (NOA) conducted a randomized phase 3 trial (NOA-08) in elderly patients. This trial demonstrated a non-inferiority of temozolomide (1 week on / 1 week off regimen) to focal radiotherapy. Importantly, hypermethylation of the *O*^{6}*-methylguanine-DNA methyltransferase* (*MGMT*) promoter was established as a very strong predictive biomarker for temozolomide sensitivity in elderly patients with malignant glioma. Of note, even though median overall survival was short (8.6 months in the temozolomide group vs. 9.6 months in the radiotherapy group), in both treatment arms a subset of patients survived considerably longer than two years [11].

Recently, several other molecular markers have been reported as being either prognostic or even predictive of benefit from specific therapeutic interventions in malignant glioma patients. In 2008, Parsons et al. discovered mutations in the isocitrate dehydrogenase 1 gene (*IDH1*) in a subset of glioblastoma patients [12]. Subsequently, mutations in either *IDH1* or *IDH2* have been identified in > 70% of WHO grade II and III gliomas and secondary glioblastomas [13]. In primary glioblastomas, *IDH* mutations are rare. Importantly, *IDH* mutations are associated with a significantly longer survival time compared to *IDH* wildtype tumors. Among grade III and IV gliomas pooled, *IDH* has a stronger prognostic impact than the WHO grade. In the same study, the authors also

found that in patients with anaplastic astrocytoma and glioblastoma aged 60 years or older, *IDH1* mutation is found in only 7.5% of the patients (11/146) [14]. Analysis of the glioblastoma epigenome revealed a distinct hypermethylator phenotype, the glioma CpG island methylator phenotype (G-CIMP), which confers a good prognosis [15]. G-CIMP positive tumors usually harbor *IDH* mutations, hence they are common among grade II and III gliomas and rare in primary glioblastomas. Genome- and epigenome-wide analysis of glioblastoma samples further revealed frequent mutations in the *histone 3.3* gene (*H3F3A*) at K27 or G34 [16]. Just like *IDH* mutations, both K27M and G34R/V mutations are associated with a distinct epigenetic signature and possibly cell of origin each [17]. In this study, tumors carrying G34 mutations show a favorable clinical course. Importantly, *H3F3A* and *IDH* mutations are mutually exclusive, suggesting that mutations in either of these genes represent different gliomagenic pathways. While the aforementioned alterations are prognostic, Agnihotri et al. recently reported on epigenetic inactivation of *alkylpurine DNA N-glycosylase* (*APNG*) as a predictive biomarker for benefit from temozolomide treatment [18]. APNG is a DNA base excision repair enzyme, which catalyzes removal of N3-methyladenine (N3-meA) and N7-methylguanine (N7-meG) from DNA, both of which can be caused by temozolomide. *APNG* expression is regulated through promoter methylation, and in patients with an unmethylated *MGMT* promoter receiving temozolomide, the subset of *APNG* negative tumors was reported to have a better prognosis. Peroxiredoxin 1 (PRDX1) is another interesting candidate, especially in the group of anaplastic astrocytomas. The *PRDX1* promoter was found to be frequently hypermethylated in oligodendroglial tumors and secondary glioblastomas carrying a deletion of 1p/19q, leading to epigenetic down-regulation of PRDX1 expression [19]. In this study, silencing of *PRDX1* in Hs683 glioma cells sensitized these cells both to temozolomide and radiotherapy *in vitro*.

The objective of our present study was to determine the prevalence and impact of recently defined biomarkers which are associated with survival, but were established in younger patients, in an elderly collective. We hypothesized that these biomarkers might help to explain the heterogeneity in survival seen in the NOA-08 population. However, as our study revealed that they are virtually absent in elderly patients, we assume that relevant molecular differences exist between malignant glioma of different age groups, which warrant further studies.

MATERIALS AND METHODS

Patients and DNA

This study comprised 126 patients of the NOA-08 collective, nine with anaplastic astrocytomas (7.1%) and 117 with glioblastomas (92.9%). All patients were 65 years or older [11].

DNA from fresh-frozen paraffin-embedded (FFPE) tissue was extracted using the Invisorb Genomic DNA Kit II (Stratec Molecular, Berlin, Germany). Before DNA extraction, specimens were histopathologically reviewed and tumor content > 80% was confirmed.

To complement our subset with a series of tumors from younger glioblastoma patients, we received bisulfite-converted DNA of ten glioblastoma patients (mean age 45 years) from the Department of Neuropathology, University of Heidelberg (W.M.).

IDH1 and *H3F3A* mutation status

Patients were screened for *IDH1* and *H3F3A* mutations by direct sequencing of PCR products or immunohistochemistry. For sequencing analysis, the following primers were used:

IDH1: 5'-CGGTCTTCAGAGAAGCCATT-3' (for.), 5'-GCAAAATCACATTATTGCCAAC-3' (rev.)

H3F3A: 5'-CATGGCTCGTACAAAGCAGA-3' (for.), 5'-CAAGAGAGACTTTGTCCCATTTTT-3' (rev.)

Sequencing was performed by GATC Biotech, Konstanz, Germany. Immunohistochemistry detecting the *IDH1R132H* mutation was performed with mouse monoclonal antibody H09 (Dianova, Hamburg, Germany) on an automated immunostainer (BenchMark, Ventana Medical Systems, Tuscon, AZ, USA).

G-CIMP status

Methylation-specific PCRs (MSP) for 8 genes were performed as previously described [15]. A sample was considered G-CIMP positive when either *DOCK5* was hypo- and 5 of the remaining 7 genes were hypermethylated or (in case of a hypermethylation of *DOCK5*) 6 out of the other 7 genes were hypermethylated. **Supplementary Table 1** lists the primers used for MSP analysis.

Quantitative high resolution DNA methylation analysis

APNG and *PRDX1* promoter methylation was screened using the MassARRAY technique (Sequenom, San Diego, CA, USA). This technology relies on detection of mass shifts, which are introduced through sequence changes following bisulfite treatment [20]. In short, 500 ng genomic DNA was bisulfite-converted using the Epitect

Bisulfite Kit (Qiagen, Hilden, Germany). For PCR amplification, HotStarTaq (Qiagen, Hilden, Germany) and the primers listed in **Supplementary Table 2** were used.

Next, DNA methylation analysis was performed on a Sequenom mass spectrometer and the results were analyzed by the Epityper software (Version 1.05, Sequenom, San Diego, CA, USA).

Illumina 450k methylation array

For genome-wide assessment of DNA methylation we used the HumanMethylation450 BeadChip (Illumina, San Diego, CA, USA). Methylation analysis of glioblastoma samples (n=22) was performed at the in-house Genomics and Proteomics Core Facility (German Cancer Research Center, Heidelberg, Germany). Methylation data of additional adult glioblastoma samples (n=74) were obtained from the database of The Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov) at June 15 2012.

TCGA collective

To assess the influence of age on survival in *IDH* wild-type patients, methylation (Illumina HumanMethylation27 BeadChip, n=294 samples and Illumina HumanMethylation450 BeadChip, n=126 samples) and clinical data were obtained from the database of The Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov) at Jan 15 2013.

Unsupervised hierarchical clustering of methylation data was performed as described previously [15,17]. Briefly, probes (i) targeting the X and Y chromosomes, (ii) containing a single nucleotide polymorphism within 5 base pairs of and including the CpG site and (iii) not mapping uniquely to the human reference genome (hg19), allowing for one mismatch, were removed. The 1500 (Illumina HumanMethylation27 BeadChip) and 8000 (Illumina HumanMethylation450 BeadChip) most variable (by SD) probes were kept and unsupervised hierarchical clustering was performed for each platform .

A logistic regression model to estimate the probability of *MGMT* methylation from Illumina HumanMethylation BeadChip data was used as described by Bady et al [21]: From the normalized methylated (m) and unmethylated (u) signal intensities, the M-value was calculated: $M\text{-value} = \log2(\max(y_{i,methyl},0)+\alpha / (\max(y_{i,unmethyl},0)+\alpha)$ [22]. The probability of *MGMT* methylation was calculated as logit(y) = 4.3215 + 0.5271 * M-value(cg12434587) + 0.9265 * M-value(cg12981137). A probability cut-off at 0.358 was used.

Statistics

MassARRAY CpG units were evaluated separately as well as averaged per amplicon. Association of quantitative MassARRAY measurements with overall survival (OS) and event-free survival (EFS) was assessed with univariate Cox PH regression models. Predictive factors were assessed with a factor vs. treatment interaction term. Proportional hazards assumption was tested for violation according to Grambsch and Therneau [23]. Risk groups were determined based on optimal cut-point analysis using the maximally selected log-rank statistic approach [24,25], which corrects for type I error inflation due to multiple testing. Survival of risk groups was estimated with the method of Kaplan and Meier. Wilcoxon rank-sum test was employed to compare median Karnofsky performance score between old and young subgroups of the TCGA data, Fisher's exact test was used to analyze the relationship between age (dichotomised as < 65 and ≥ 65 years) and *MGMT* methylation, extent of surgery and treatment, respectively. Univariable p values were adjusted for multiple testing using Benjamini-Hochberg correction in order to control the false discovery rate [26]. All tests were two-sided. P values below 0.05 were considered statistically significant. Analyses were carried out using software R Version 2.14 [27].

RESULTS

Study population

In total, 126 patients of the NOA-08 trial (termed NOA-08 biomarker cohort) were analyzed. **Table 1** lists the patient characteristics of this study collective and the NOA-08 collective. Patients with a resection rather than a biopsy are overrepresented in this cohort due to the requirement of a sufficient amount of tissue. *MGMT* promoter methylation status was similar between both groups. Median EFS times were comparable between our study population (4.4 months, 95% CI 3.7-5.4 months) and the entire NOA-08 collective (4.1 months, 95% CI 3.7-4.5 months), while median overall survival was higher (11.2 [95% CI 9.5-13.6] vs. 8.9 [95% CI 8.0-9.9] months). Long-term OS data are demonstrating a group of patients with a considerably longer than average survival mainly in the temozolomide arm (**Figure 1a**). As expected from the increased median OS, these patients are overrepresented in our cohort due to enrichment of patients having undergone resection instead of biopsy. **Supplementary Table 3** lists the EFS and OS data for all 126 patients included in this study.

In the NOA-08 trial, histology (anaplastic astrocytoma vs. glioblastoma) did not significantly influence event-free or overall survival [11]. This is recapitulated in the present biomarker cohort, where histology has no influence on event-free or overall survival. Kaplan-Meier plots are depicted in **Supplementary Figure 1**.

Importantly, the predictive value of *MGMT* promoter methylation for response to temozolomide is recapitulated in this NOA-08 biomarker cohort (interaction p=0.03 (OS) and <0.001 (EFS)). In a Cox regression model, *MGMT* promoter hypermethylation significantly prolonged EFS and OS in patients treated with temozolomide ($p < 0.0001$ and p=0.0014, respectively), while in the radiotherapy group, the influence of *MGMT* promoter methylation on survival was not significant (p=0.34 for EFS and p=0.14 for OS) (**Figure 1b,c**).

IDH1 mutation, G-CIMP status and *H3F3A* mutations

In this study cohort, three patients carried an *IDH1* mutation in their tumor tissue as determined by sequencing. Of these, two patients had glioblastomas (out of 117 glioblastoma patients) and one had an anaplastic astrocytoma (out of 9 patients). The two glioblastoma patients had an overall survival of 582 and 924 days, respectively. This is above the median OS (272 (231-315 days) in the NOA-08 group, whereas the patient with an anaplastic astrocytoma had shorter than median survival (patient 37, OS 196 days). Notably, *MGMT* promoter analysis revealed hypermethylation in all 3 patients. On the other hand, only patient 110 received temozolomide as first-line treatment (**Table 2**).

To expand the sample size, we performed IDH1 immunohistochemistry on another 50 patients from the NOA-08 trial of whom only unstained paraffin-embedded slides, but no tumor DNA for further analysis, were available. In this group, no *IDH1*(R132H) mutant tumor was detected. In summary, the low frequency of *IDH1* mutations precludes a predictive role of this marker in elderly patients with glioblastoma, although individual patients may have a better course than the average.

In line with earlier reports demonstrating a strong correlation between *IDH1* mutations and G-CIMP, the eight-gene MSP panel revealed that these three *IDH1* mutant patients were also G-CIMP positive. In the remaining *IDH1* wildtype group of 123 samples, 4 samples could not be analyzed due to insufficient DNA left for bisulfite conversion and the other 119 were G-CIMP negative.

To screen our biomarker cohort for the recently described *H3F3A* K27M and G34R/V mutations, we amplified and sequenced a 170 bp fragment spanning the two mutation sites of the *H3F3A* gene in all 103 patients for which enough suitable DNA was left. We detected neither K27M nor G34R/V mutations in our cohort.

APNG and *PRDX1* methylation

CpG methylation was assessed by MassARRAY technology. In total, 99 (*APNG*) and 73 (*PRDX1*) tumors of the 126 sample cohort were analyzed. Methylation levels across the 20 examined CpGs (measured as 13 distinct CpG units) in the promoter / intron 1 of the *APNG* locus were low apart from a few exceptions (median 6%, interquartile range 5-9%). The heatmap of *APNG* methylation is depicted in **Figure 2a**. Mean quantitative CpG methylation levels of APNG were not associated with EFS (p=0.54) or OS (p=0.61). Similarly, discrimination between *APNG* methylated and *APNG* nonmethylated patients at a cut-off value of 10% (EFS, p=0.71) and 3% (OS, p=0.42), respectively, which optimally separated the curves, did not yield a significant risk stratification, independent of treatment. When analyzing the predictive effect of *APNG* methylation in patients with a nonmethylated *MGMT* promoter who were treated with temozolomide, the low methylation levels precluded identification of a biologically meaningful cutpoint (7.2% for OS and 8.5% for EFS, respectively).

Since APNG has been proposed as a biomarker predictive for the benefit from temozolomide with average methylation levels varying between 30-40% (APNG expressers) and 70-80% (APNG non-expressers) [18], we next aimed at investigating reasons for that discrepancy to our findings. To exclude *APNG* as a biomarker relevant only for younger patients, we also did MassARRAY analyses on 10 patients with an average age of 45 years. This analysis yielded the same homogenously low methylation level as in the NOA-08 biomarker cohort (data not shown). In addition, Illumina 450k methylation arrays were performed for 22 patients from the NOA-08 biomarker collective. Further, samples from the TCGA project, which were analyzed on the same Illumina 450k

platform (n=74, mean age 61 years, range 23 – 85 years), have been included. Two probes (cg05397937 and cg15768556) surveyed in total 3 CpGs, which are also included in both our MassARRAY amplicon and bisulfite sequencing performed by Agnihotri and colleagues (see **Figure 2c**). Consistent with the MassARRAY findings homogenously low levels of methylation are demonstrated across all 96 samples (< 0.2, see **Figure 2d,e**). Even though 5 TCGA samples clustered into the G-CIMP group [15], they did not show increased levels of *APNG* methylation compared to non-G-CIMP samples. Of note, comparing MassARRAY data and 450k data for the NOA-08 patients of whom both datasets were available showed a good agreement between both methods.

Promoter methylation analysis of *PRDX1* revealed moderately low methylation with most samples exhibiting a mean methylation between 10 and 20% across the 6 CpGs (measured as 5 distinct CpG units) examined (**Figure 2b**). Only 8 out of 73 samples had a mean methylation > 30%. *PRDX1* methylation did not show an association with EFS or OS, neither in the whole study population nor by treatment.

Age-related survival differences

Given the paucity of positive prognostic factors in older glioblastoma patients, we sought to determine whether the relative absence of these known factors alone might explain the survival differences seen between different age groups using the TCGA data set. We determined G-CIMP (as a surrogate marker for *IDH* mutation) and *MGMT* promoter methylation status from Illumina HumanMethylation BeadChip data as previously described[15,17,21] and complemented these molecular data with clinical information from the TCGA database. In total, 170 patients had a complete clinical and molecular dataset. In this cohort, we detected 18 patients with a hypermethylator phenotype (10.5%), of which only 2 were older than 65 years. G-CIMP status was associated with a significantly prolonged survival (median overall survival 22.7 [95% CI 8.4-not reached] vs. 15.9 [95% CI 14.1-17.6] months, logrank p=0.0085, **Supplementary Figure 2a**). *MGMT* promoter hypermethylation as predicted through a logistic regression model occurred in 70 cases (41%) and was associated with improved median overall survival in patients who initially received a combined radio-chemotherapy (19.7 [95% CI 15.7-23.9] vs. 14.5 [95% CI 12.2-16.6] months, logrank p=0.0288, **Supplementary Figure 2b**). For further analysis, we excluded G-CIMP positive patients. **Table 3** summarizes the baseline characteristics of both groups (G-CIMP negative patients < 65 and ≥ 65 years of age). Notably, the two groups showed no significant differences with regards to relevant prognostic or predictive parameters, albeit there was a trend towards a higher Karnofsky performance score in the younger cohort. Despite the balance between the groups, older patients had a significantly shorter overall survival (12.5 [95% CI 7.6-14.4] vs. 17.6 [95% CI 15.7-20.3] months, logrank p=0.0007, **Figure 3a**). To account for a potential confounding effect of the Karnofsky performance score, we

performed a multivariate Cox regression analysis including age (as a dichotomous variable < 65 vs. ≥ 65 years of age) and Karnofsky performance score (as a categorical covariate). Conforming the above result, this analysis yielded a hazard ratio of 2.25 (95% CI 1.46-3.47, $p < 0.001$) for patients ≥ 65 years of age adjusted for Karnofsky performance score.

DISCUSSION

Elderly patients will soon account for more than half of all glioblastoma patients in the Western countries [2]. Despite this development, elderly patients are still underrepresented in clinical trials, leaving the standard care for this population currently ill-defined [3]. There is increasing evidence to suggest fundamental molecular differences between malignant gliomas of different age groups. In 2004, Batchelor et al. demonstrated age-dependent effects of the prognostic impact of key genomic alterations (*TP53* mutation, *CDK2NA*/*p16* deletion and loss of chromosome 1p) in glioblastoma [28]. Recently, analysis of common genomic aberrations in glioblastoma (*TP53* mutation, EGFR amplification, EGFR vIII mutant, *PTEN* deletion and *IDH1* mutation) have revealed distinctive differences in the distribution of these aberrations in young adults (19 – 40 years of age) and patients older than 40 years [29]. In the pediatric population, somatic mutations in the H3.3-ATRX-DAXX chromatin remodeling pathway have been discovered in 44% of glioblastomas [16]. Tumors carrying a mutation in this pathway were associated with a distinct gene expression profile. It has recently been shown that tumors with *H3.3* mutations have indeed a distinct methylation, gene expression, mutation and copy number variation profile and possibly cell of origin [17]. Molecular analysis of glioblastomas across the age continuum showed that *H3.3* mutations (K27M and G34R/V) predominantly occurred in children and young adults and *IDH* mutations in young adults, while older patients mostly were classified into the remaining three subtypes (mesenchymal subtype, RTK 1 "PDGFRA" and RTK 2 "classic"). In agreement with this, *IDH* mutations are very rare in patients with malignant gliomas above the age of 60 [14]. The lack of *IDH* mutations in these tumors might partially contribute to the worse prognosis of elderly patients with malignant gliomas, even though the analysis of the TCGA collective suggests that also other factors play a role in these age-related survival differences. Along this line, we found only 3 *IDH1* mutated patients in our cohort of 126 patients, and no *H3F3A* mutations. In agreement with the recently discovered causative role of *IDH* mutations in epigenetic remodeling resulting in a G-CIMP phenotype, our 3 *IDH1* mutated samples proved G-CIMP positive as well [30,31]. With *IDH1* mutations accounting for more than 90% of *IDH* mutations in glioma[13], the causality between *IDH* mutation and the G-CIMP phenotype and the lack of a G-CIMP positive tumor in the remaining 119 *IDH1* wildtype samples, we decided against testing for *IDH2* mutations. Importantly, while the two glioblastoma patients carrying an *IDH1* mutation and G-CIMP had an overall survival above average (see **Table 2**), 15 *IDH* wildtype / G-CIMP negative patients in this study population had a comparable or even longer overall survival than patient 110, who is *IDH* mutated / G-CIMP positive. Of these 15 patients one patient displayed a longer overall survival than the *IDH* mutated / G-CIMP positive patient 125 (see **Supplementary Table 3**).

The rationale for the further selection the biomarkers investigated in this study was to explain the survival heterogeneity seen in the NOA-08 trial population, where a group of patients had a considerably longer survival than average[11]. *MGMT* promoter methylation alone is not sufficient to account for this. In glioblastoma, key chromosomal, genetic and epigenetic aberrations have been defined, including *EGFR* amplification, *TP53* mutation, *CDK4* amplification, *CDKN2A* homozygous deletion or *IDH* mutations [12,32]. However, with the notable exception of *IDH* mutations [13], these molecular aberrations have no significant effect on survival [33]. With regard to the aim of our study, we therefore limited the selection of biomarkers to prognostically relevant markers.

A recent report demonstrated a role for the DNA repair enzyme APNG in conferring resistance to temozolomide in glioblastoma [18]. Patients without hypermethylation of the *MGMT* promoter were subgrouped into APNG expressers and non-expressers (assessed by immunohistochemistry), where tumors that expressed APNG had a worse prognosis when treated with a temozolomide-containing regimen. To investigate the role of promoter methylation in regulation of APNG expression, these authors performed bisulfite sequencing of an approximately 200 bp fragment located in the promoter / intron 1 region of *APNG*. Comparing APNG expressers and non-expressers, differences in mean methylation levels across the investigated fragment (37 ± 5% for expressers and 77 ± 6% for non-expressers) were found. *In vitro* treatment of human glioblastoma cell lines with the demethylating agent 5-azacytidine resulted in up-regulation of APNG mRNA levels. This led the authors to propose that APNG expression is regulated through promoter hypermethylation. As the NOA-08 study and the present biomarker cohort clearly demonstrate the predictive effect of *MGMT* promoter hypermethylation described earlier [34], we sought to determine if *APNG* methylation also has the predictive value as proposed before. In contrast to Agnihotri and colleagues, we performed MassARRAY analysis of the same fragment, a well established technique for quantitative assessment of methylation [20]. Surprisingly, we only detected <20% levels of *APNG* promoter methylation across all samples and CpG units (**Figure 2**) and did not observe the separation of patients into two groups. Since methylation is known to be at least partially age-dependent [35], we expanded our MassARRAY analysis to include ten younger glioblastoma patients (mean age 45 years) and obtained similar results. Our findings were confirmed through a technically and (partly) biologically independent analysis of Illumina 450k methylation arrays of 22 NOA-08 samples and 74 TCGA samples. The low methylation of *APNG* is no age-specific effect, as evidenced by the TCGA samples, which include patients aged between 23 and 85 years (mean age 61 years) and showed homogenously low methylation levels across all samples. Furthermore, the methylation-specific predictive effect of *MGMT* promoter methylation is highly significant in both our subset and the whole NOA-08 cohort. A possible explanation for the conflicting results

regarding *APNG* methylation may lie in the use of the non-quantitative bisulfite sequencing, which requires clonal amplification prior to sequencing, thus introducing an additional step for potential bias. Our *APNG* amplicon was marginally larger than that examined by Agnihotri et al. (233 bp vs. 193 bp), yet addressed exactly the same CpGs (**Figure 2c**). We carefully re-inspected our amplicon sequence with respect to possible SNPs in primer sequences and GC-content, being 41% after bisulfite treatment in case all CpGs would be methylated, and found no obvious hint for any PCR bias in our setting. Further studies will be required to assess the role of epigenetic regulation in *APNG* expression.

Analysis of *PRDX1* promoter methylation, a novel marker for sensitivity towards temozolomide or radiotherapy, yielded no significant effect on survival. This is in line with the original report on *PRDX* methylation, which suggested that hypermethylation mostly occurs in oligodendroglial tumors and secondary glioblastomas [19].

Although the biomarker cohort analyzed in the present study was representative for the NOA-08 study population (**Table 1**), subgroup analyzes have well-known limitations. Further some DNA samples extracted from FFPE tissue proved to be too fragmented to allow for PCR amplification of the desired MassARRAY amplicons (each > 200 bp), explaining the discrepancy in analyzed samples between G-CIMP MSP (n=126, with average amplicon size of 100 bp) and MassARRAY (n=99 and 73 samples for *APNG* and *PRDX1*, respectively).

To assess the influence of age on survival, we analyzed survival in a large cohort from the TCGA, which was well matched for all relevant prognostic factors. However, the median survival times of the TCGA collective are subject to a selection bias, e.g. due to the tissue requirements for molecular analysis (exemplified by a 90% resection rate, **Table 3** versus 60% in NOA-08 [11]) and thus cannot be externally compared, e.g. to the NOA-08 survival times. Nonetheless, in the intragroup comparison, patients aged ≥ 65 years still had significantly shorter overall survival times than their younger counterparts.

In summary, favorable prognostic biomarkers such as *IDH* or *H3F3A* mutation, G-CIMP or *PRDX1* methylation are virtually absent in malignant astrocytic tumors of the elderly which may partially account for the worsened prognosis of these patients. However, even in G-CIMP negative (and hence *IDH* wild-type) tumors, which are matched for known prognostic factors, older patients have a significantly shorter overall survival. On the other hand, several long-term surviving patients in our cohort exist which lack the aforementioned molecular markers and sometimes even *MGMT* promoter hypermethylation. These two observations strongly hint at the existence of so-far unknown prognostic factors in these patients. Further studies are necessary to broaden our insight into the molecular aberrations in elderly patients in order to step-wise replace chronological age as the most important negative prognostic marker and potentially therapy-decisive variable in malignant astrocytomas with defined and hopefully actionable molecular mechanisms.

FUNDING

The current research was supported within the Brain Tumor Network (BTNplus; Subproject 6) of the National Genome Research Network (NGFNplus) by the Federal Ministry of Education and Research (BMBF), and the charitable Hertie Foundation.

ACKNOWLEDGEMENTS

We are indebted to the patients and their families for agreeing to participate in the NOA-08 trial, as well as to the nurses and data managers for their collaboration. We thank Matthias Schick and Roger Fischer from the DKFZ Gene Core Facility for performing the microarray analyses to a very high standard. The NOA-08 study has been awarded the quality label A by the German Cancer Society. Alcedis (Giessen, Germany) served as CRO for monitoring and data collection. The first round of *MGMT* testings reported in this study was done as a free scientific service by MDx Health (Liège, Belgium).

REFERENCES

1. Iwamoto FM, Reiner AS, Panageas KS, Elkin EB, Abrey LE. Patterns of care in elderly glioblastoma patients. *Annals of neurology.* Dec 2008;64(6):628-634.
2. Weller M, Platten M, Roth P, Wick W. Geriatric neuro-oncology: from mythology to biology. *Current opinion in neurology.* Dec 2011;24(6):599-604.
3. Laperriere N, Weller M, Stupp R, et al. Optimal management of elderly patients with glioblastoma. *Cancer treatment reviews.* Jun 19 2012.
4. Stupp R, Mason WP, van den Bent MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *The New England journal of medicine.* Mar 10 2005;352(10):987-996.
5. Sijben AE, McIntyre JB, Roldan GB, et al. Toxicity from chemoradiotherapy in older patients with glioblastoma multiforme. *Journal of neuro-oncology.* Aug 2008;89(1):97-103.
6. Lawrence YR, Li XA, el Naqa I, et al. Radiation dose-volume effects in the brain. *International journal of radiation oncology, biology, physics.* Mar 1 2010;76(3 Suppl):S20-27.
7. Ruben JD, Dally M, Bailey M, Smith R, McLean CA, Fedele P. Cerebral radiation necrosis: incidence, outcomes, and risk factors with emphasis on radiation parameters and chemotherapy. *International journal of radiation oncology, biology, physics.* Jun 1 2006;65(2):499-508.
8. Keime-Guibert F, Chinot O, Taillandier L, et al. Radiotherapy for glioblastoma in the elderly. *The New England journal of medicine.* Apr 12 2007;356(15):1527-1535.
9. Chinot OL, Barrie M, Frauger E, et al. Phase II study of temozolomide without radiotherapy in newly diagnosed glioblastoma multiforme in an elderly populations. *Cancer.* May 15 2004;100(10):2208-2214.
10. Gallego Perez-Larraya J, Ducray F, Chinot O, et al. Temozolomide in elderly patients with newly diagnosed glioblastoma and poor performance status: an ANOCEF phase II trial. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* Aug 1 2011;29(22):3050-3055.
11. Wick W, Platten M, Meisner C, et al. Temozolomide chemotherapy alone versus radiotherapy alone for malignant astrocytoma in the elderly: the NOA-08 randomised, phase 3 trial. *The lancet oncology.* May 10 2012.
12. Parsons DW, Jones S, Zhang X, et al. An integrated genomic analysis of human glioblastoma multiforme. *Science.* Sep 26 2008;321(5897):1807-1812.
13. Yan H, Parsons DW, Jin G, et al. IDH1 and IDH2 mutations in gliomas. *The New England journal of medicine.* Feb 19 2009;360(8):765-773.
14. Hartmann C, Hentschel B, Wick W, et al. Patients with IDH1 wild type anaplastic astrocytomas exhibit worse prognosis than IDH1-mutated glioblastomas, and IDH1 mutation status accounts for the unfavorable prognostic effect of higher age: implications for classification of gliomas. *Acta neuropathologica.* Dec 2010;120(6):707-718.
15. Noushmehr H, Weisenberger DJ, Diefes K, et al. Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer cell.* May 18 2010;17(5):510-522.
16. Schwartzentruber J, Korshunov A, Liu XY, et al. Driver mutations in histone H3.3 and chromatin remodelling genes in paediatric glioblastoma. *Nature.* Feb 9 2012;482(7384):226-231.
17. Sturm D, Witt H, Hovestadt V, et al. Hotspot Mutations in H3F3A and IDH1 Define Distinct Epigenetic and Biological Subgroups of Glioblastoma. *Cancer cell.* Oct 16 2012;22(4):425-437.
18. Agnihotri S, Gajadhar AS, Ternamian C, et al. Alkylpurine-DNA-N-glycosylase confers resistance to temozolomide in xenograft models of glioblastoma multiforme and is associated with poor survival in patients. *The Journal of clinical investigation.* Jan 3 2012;122(1):253-266.
19. Dittmann LM, Danner A, Gronych J, et al. Downregulation of PRDX1 by promoter hypermethylation is frequent in 1p/19q-deleted oligodendroglial tumours and increases radio- and chemosensitivity of Hs683 glioma cells in vitro. *Oncogene.* Dec 12 2011.

20. Ehrich M, Nelson MR, Stanssens P, et al. Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. *Proceedings of the National Academy of Sciences of the United States of America.* Nov 1 2005;102(44):15785-15790.

21. Bady P, Sciuscio D, Diserens AC, et al. MGMT methylation analysis of glioblastoma on the Infinium methylation BeadChip identifies two distinct CpG regions associated with gene silencing and outcome, yielding a prediction model for comparisons across datasets, tumor grades, and CIMP-status. *Acta neuropathologica.* Oct 2012;124(4):547-560.

22. Du P, Zhang X, Huang CC, et al. Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. *BMC bioinformatics.* 2010;11:587.

23. Grambsch PM, Therneau T. Proportional hazards tests and diagnostics based on weighted residuals. *Biometrika.* 1994;81:515-526.

24. Lausen B, Hothorn T, Bretz F, Schumacher M. Optimally Selected Prognostic Factors. *Biometrical Journal.* 2004;46:364-374.

25. Lausen B, Schumacher M. Maximally Selected Rank Statistics. *Biometrics.* 1992;48:73-85.

26. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B.* 1995;57:289-300.

27. R Development Core Team. *R: A language and environment for statistical computing.* Vienna, Austria: R Foundation for Statistical Computing; 2011.

28. Batchelor TT, Betensky RA, Esposito JM, et al. Age-dependent prognostic effects of genetic alterations in glioblastoma. *Clinical cancer research : an official journal of the American Association for Cancer Research.* Jan 1 2004;10(1 Pt 1):228-233.

29. Jha P, Suri V, Singh G, et al. Characterization of molecular genetic alterations in GBMs highlights a distinctive molecular profile in young adults. *Diagnostic molecular pathology : the American journal of surgical pathology, part B.* Dec 2011;20(4):225-232.

30. Lu C, Ward PS, Kapoor GS, et al. IDH mutation impairs histone demethylation and results in a block to cell differentiation. *Nature.* Mar 22 2012;483(7390):474-478.

31. Turcan S, Rohle D, Goenka A, et al. IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. *Nature.* Mar 22 2012;483(7390):479-483.

32. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature.* Oct 23 2008;455(7216):1061-1068.

33. Weller M, Felsberg J, Hartmann C, et al. Molecular predictors of progression-free and overall survival in patients with newly diagnosed glioblastoma: a prospective translational study of the German Glioma Network. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* Dec 1 2009;27(34):5743-5750.

34. Hegi ME, Diserens AC, Gorlia T, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *The New England journal of medicine.* Mar 10 2005;352(10):997-1003.

35. Christensen BC, Houseman EA, Marsit CJ, et al. Aging and environmental exposures alter tissue-specific DNA methylation dependent upon CpG island context. *PLoS genetics.* Aug 2009;5(8):e1000602.

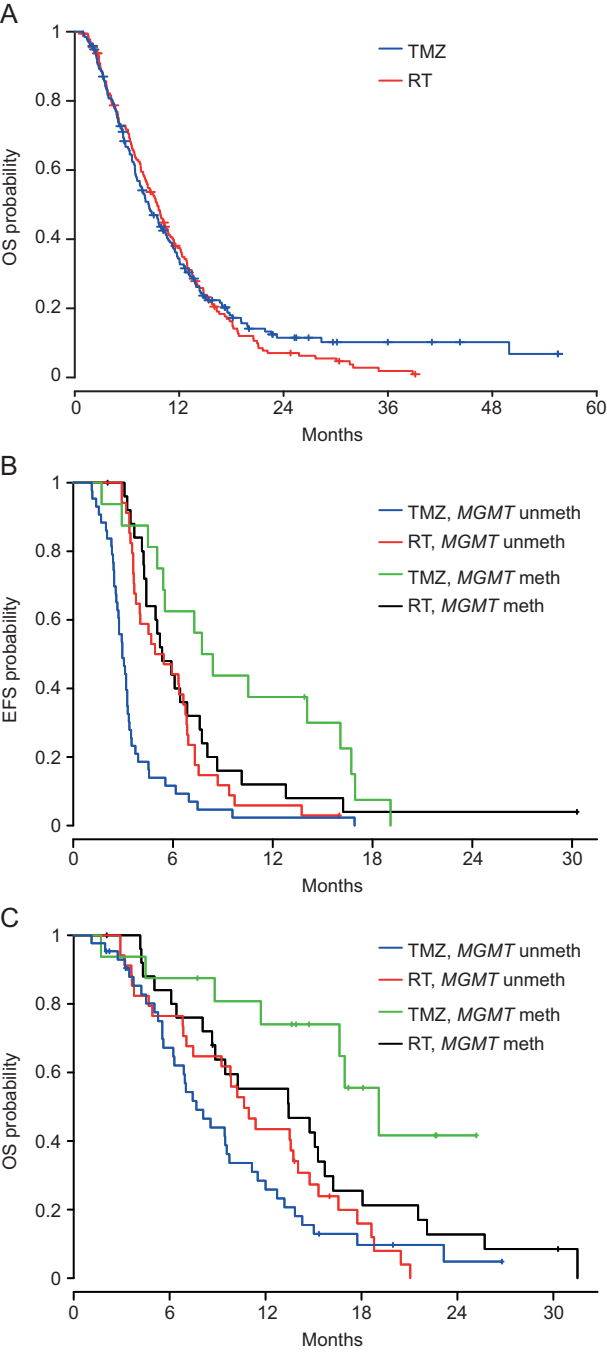
FIGURE CAPTIONS

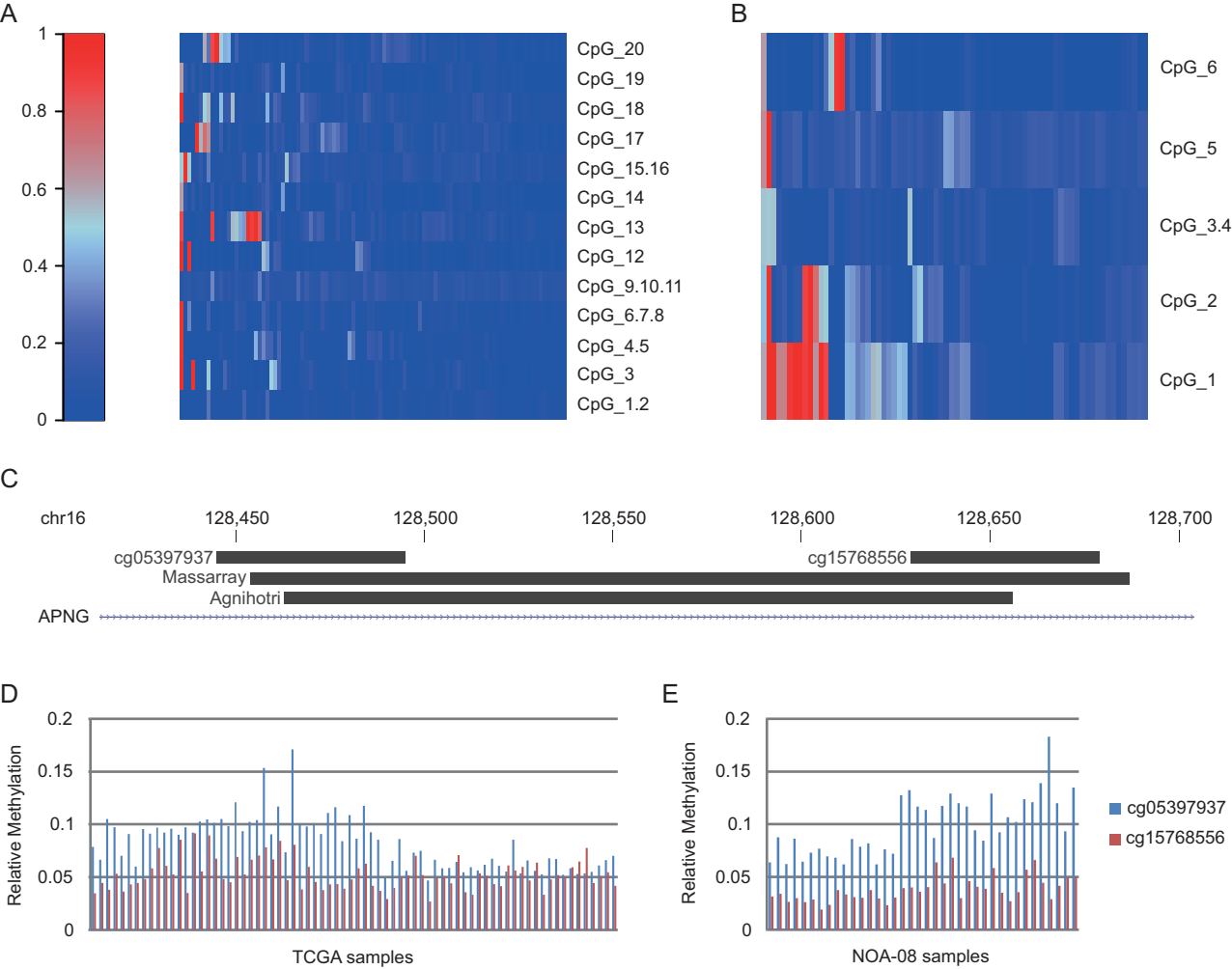
Figure 1. Survival by *MGMT* promoter methylation status and treatment. (A) Unlimited Kaplan-Meier plots for OS of the radiotherapy (RT) and temozlomide (TMZ) group, respectively. (B-C) The Kaplan-Meier plots depict the relationship between *MGMT* promoter methylation and treatment for EFS (B) and OS (C).

Figure 2. Analysis of *APNG* and *PRDX1* methylation. (A-B) Heatmaps of *APNG* (A) and *PRDX1* (B) promoter methylation. Each column represents a sample, each row a CpG unit. Methylation values are ranging from 0 (totally unmetylated) to 1 (fully methylated) and are color-coded, the legend is shown left of the heatmaps. (C) Representation of the spatial relationship between the assessed CpGs of *APNG*. (D-E) Display of average methylation levels (beta values) for the TCGA (D) and NOA-08 (E) samples.

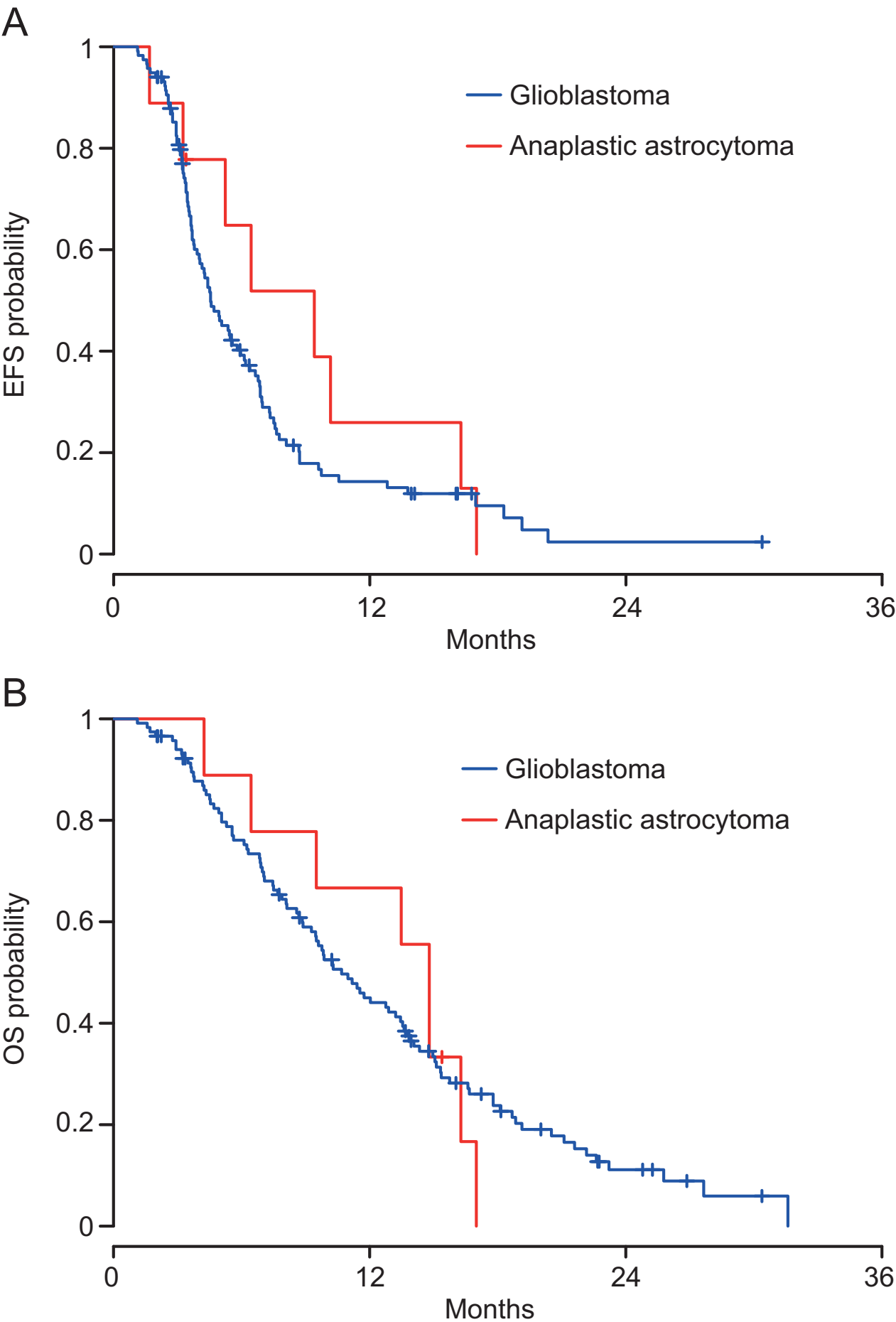
Figure 3. Survival by age in the TCGA collective. (A) Overall survival of G-CIMP negative patients, stratified by age (< 65 vs. ≥ 65 years of age).

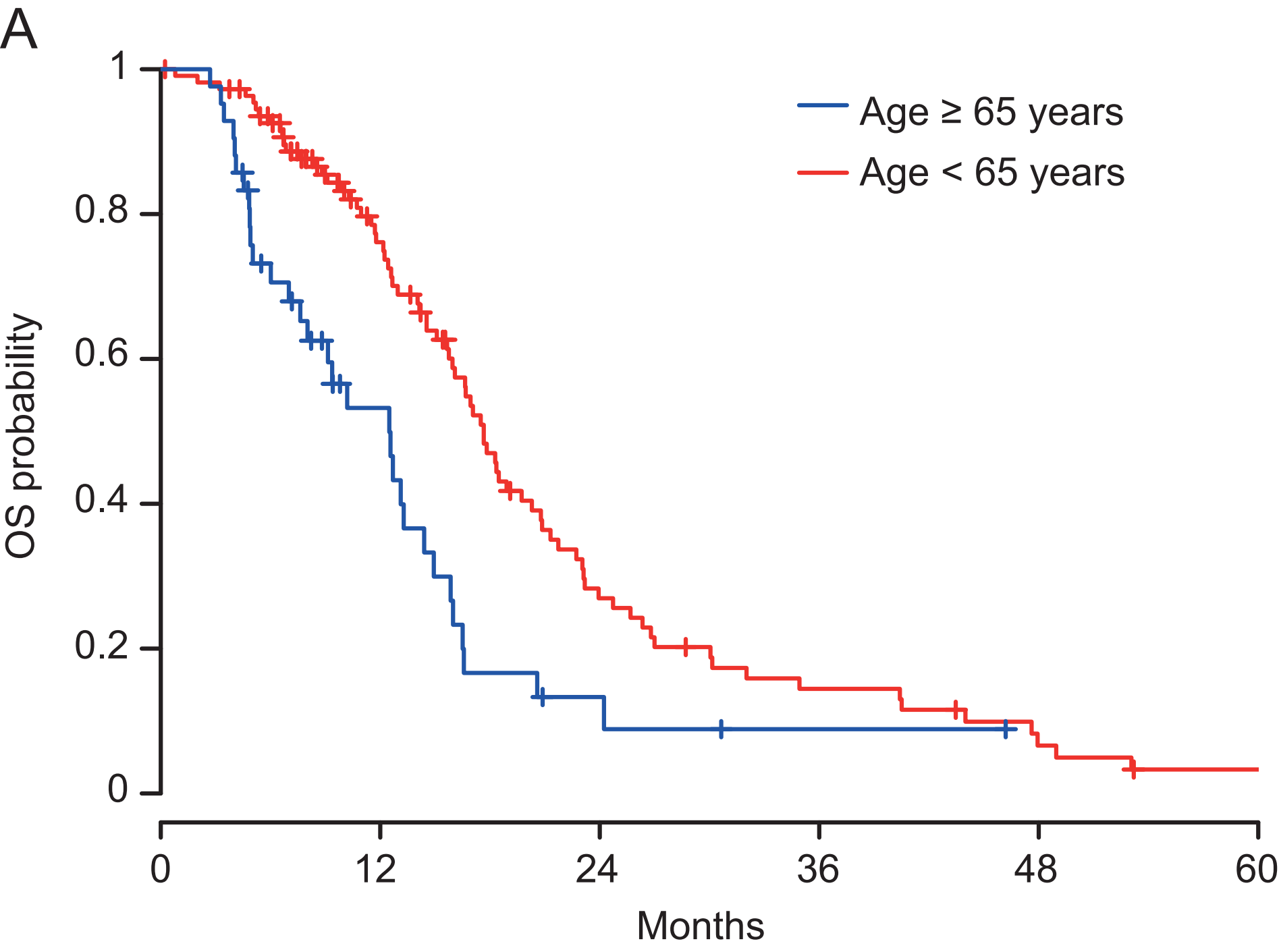
RT, Radiotherapy; TMZ; Temozolomide


A
OS probability
1
0.8
0.6
0.4
0.2
0
0
12
24
36
48
60
Months
TMZ
RT
B
EFS probability
1
0.8
0.6
0.4
0.2
0
0
6
12
18
24
30
Months
TMZ, MGMT unmeth
RT, MGMT unmeth
TMZ, MGMT meth
RT, MGMT meth
C
OS probability
1
0.8
0.6
0.4
0.2
0
0
6
12
18
24
30
Months
TMZ, MGMT unmeth
RT, MGMT unmeth
TMZ, MGMT meth
RT, MGMT meth

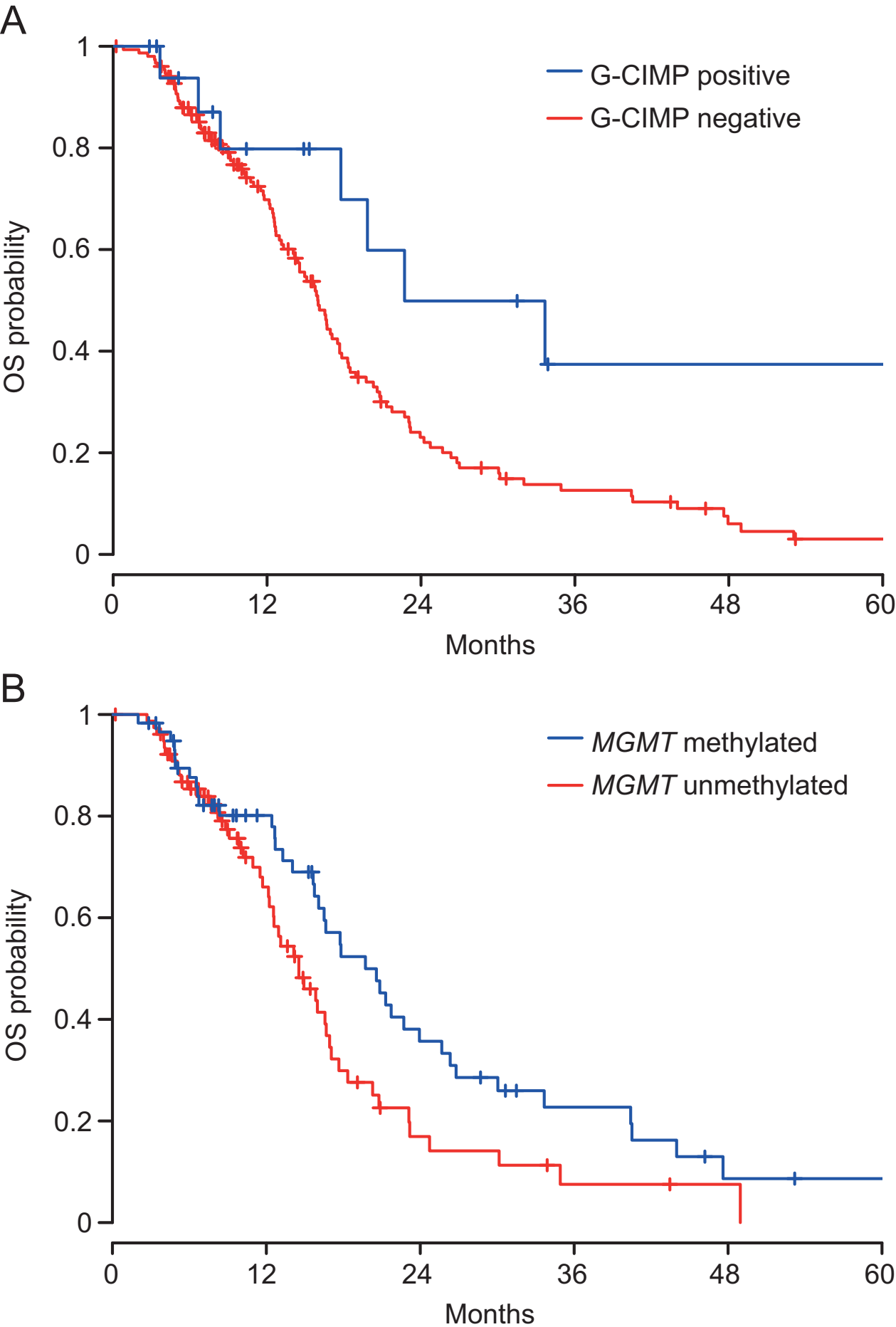

A
1
0.8
0.6
0.4
0.2
0
CpG_20
CpG_19
CpG_18
CpG_17
CpG_15.16
CpG_14
CpG_13
CpG_12
CpG_9.10.11
CpG_6.7.8
CpG_4.5
CpG_3
CpG_1.2
B
CpG_6
CpG_5
CpG_3.4
CpG_2
CpG_1
C
chr16
128,450
128,500
128,550
128,600
128,650
128,700
cg05397937
cg15768556
Massarray
Agnihotri
APNG
D
Relative Methylation
0.2
0.15
0.1
0.05
0
TCGA samples
E
Relative Methylation
0.2
0.15
0.1
0.05
0
NOA-08 samples
cg05397937
cg15768556

SUPPLEMENTARY FIGURE 1




A
1
0.8
0.6
0.4
0.2
0
OS probability
Age ≥ 65 years
Age < 65 years
0
12
24
36
48
60
Months

SUPPLEMENTARY FIGURE 2



SUPPLEMENT

Supplementary Figure 1. Survival by histology. (A-B) Kaplan-Meier plots for event-free (A) and overall survival (B) grouped by histology (anaplastic astrocytoma vs. glioblastoma).

Supplementary Figure 2. Survival in the TCGA collective by molecular markers. (A) Kaplan-Meier plot for overall survival, stratified by G-CIMP Status. (B) Overall survival depicted in patients who received radiotherapy plus temozolomide as first-line treatment, stratified by *MGMT* promoter methylation status.

Supplementary Table 1: List of primers used for detecting the G-CIMP status

Name	**Sequence**
M1-ANKRD43-for	CGGTGTTGGGTTTTTTGTAGGAGTACGGC
M1-ANKRD43-rev	TCGCCGACATCGAACAACGA
U1-ANKRD43-for	TGGTGTTGGGTTTTTTGTAGGAGTATGGT
U1-ANKRD43-rev	TCACCAACATCAAACAACAA
M1-DOCK5-for	CGGTTCGCGGAGTTTAGC
M1-DOCK5-rev	AACTACTACAACTCCTCGAACTCCG
U1-DOCK5-for	TGGTTTGTGGAGTTTAGT
U1-DOCK5-rev	AACTACTACAACTCCTCAAACTCCA
M1-HFE-for	TTTTTGATGTTTTTGTAGATCGCG
M1-HFE-rev	CGCGCCCCTAATTCGC
U1-HFE-for	TTTTTGATGTTTTTGTAGATTGTG
U1-HFE-rev	CACACCCCTAATTCAC
M1-LGALS3-for	GCGGAGTTTCGTGGGTTTCG
M1-LGALS3-rev	AATAACCAAACTACGACTCGTCACC
U1-LGALS3-for	GTGGAGTTTTGTGGGTTTTG
U1-LGALS3-rev	AATAACCAAACTACAACTCATCACC
M1-MAL-for	GTTCGGTGTAGGATTTTAGCGTC
M1-MAL-rev	ATCTACAATAAAAAATAAAACCGACCG
U1-MAL-for	GTTTGGTGTAGGATTTTAGTGTT

U1-MAL-rev	ATCTACAATAAAAAATAAAACCAACCA
M1-RHOF-for	GTTGCGGGTTTCGGGTAATGGATGTT
M1-RHOF-rev	ACCGCAACCGCCGTCGCCCACG
U1-RHOF-for	GTTGTGGGTTTTGGGTAATGGATGTT
U1-RHOF-rev	ACCACAACCACCATCACCCACA
M1-FAS1-for	AGGAACGTTTTGGGATAGGAA
M1-FAS1-rev	CAACTTAACCTACGCGCGAAT
U1-FAS1-for	AGGAATGTTTTGGGATAGGAA
U1-FAS1-rev	CAACTTAACCTACACACAAAT
M1-FAS2-for	GGGTAGGAGGTCGGTTTTCG
M1-FAS2-rev	TTCGTTACACAAATAAACATTCCTATCC
U1-FAS2-for	GGGTAGGAGGTTGGTTTTTG
U1-FAS2-rev	TTCATTACACAAATAAACATTCCTATCC

Supplementary Table 2: MassARRAY primer for *APNG* and *PRDX1* promoter methylation

Name	**Sequence (5'-3')**
APNG_for	aggaagagagGGGTAGAGTTAGAGTATAGGTTAAGGG
APNG_rev	cagtaatacgactcactatagggagaaggctCTAATCTTTAACAACACCTAAATCCTCCTAAC
PRDX1_for	aggaagagagTGAAGGAAGTTATTTAAGTTATGAGGG
PRDX1_rev	cagtaatacgactcactatagggagaaggctAAACCAAAATTCTCTTTACTTCCAAA

Supplementary Table 3: Survival data for all 126 patients included in this study

Patient ID	EFS (days)	OS (days)
1	34	34
2	48	48
3	52	52
4	60	60
5	62	62
6	63	63
7	68	70
8	47	84
9	89	89
10	89	89
11	97	97
12	84	98
13	75	99
14	95	102
15	106	106
16	110	110
17	111	111
18	114	114
19	115	115
20	112	127
21	51	129
22	129	129
23	94	132
24	137	137
25	138	138
26	143	143
27	150	150

28	154	154
29	74	154
30	42	161
31	89	169
32	107	169
33	73	171
34	186	186
35	102	190
36	35	192
37	**196**	**196**
38	208	208
39	209	209
40	71	210
41	212	212
42	75	214
43	105	215
44	90	227
45	110	228
46	99	234
47	236	236
48	78	240
49	246	246
50	79	247
51	228	261
52	264	264
53	180	265
54	222	269
55	130	270
56	122	282
57	84	288

58	99	289
59	134	289
60	292	292
61	97	297
62	223	299
63	108	300
64	193	311
65	181	312
66	163	313
67	206	325
68	194	334
69	188	340
70	112	347
71	100	351
72	165	357
73	78	366
74	83	388
75	176	392
76	97	402
77	209	409
78	309	410
79	211	412
80	123	414
81	256	416
82	419	419
83	223	421
84	169	422
85	424	424
86	103	428
87	139	436

88	154	449
89	159	450
90	286	450
91	265	455
92	119	458
93	126	460
94	99	466
95	166	467
96	103	468
97	232	479
98	488	488
99	495	495
100	296	505
101	321	507
102	517	517
103	429	524
104	90	541
105	230	541
106	105	551
107	168	552
108	202	568
109	265	573
110	**582**	**582**
111	93	609
112	103	624
113	138	642
114	134	657
115	236	674
116	619	688
117	510	690

118	490	692
119	516	706
120	98	754
121	89	768
122	151	784
123	81	817
124	556	841
125	**924**	**924**
126	390	961

Bold patients are *IDH* mutated / G-CIMP positive

TABLES

Table 1: Comparison of patient characteristics of this study population and the NOA-08 collective

	This study population (n=126)	NOA-08 (n=373)
Histology		
Anaplastic astrocytoma	9 (7%)	40 (11%)
Glioblastoma	117 (93%)	331 (89%)
Not confirmed	0	2
Treatment		
Temozolomide	62 (49%)	195 (52%)
Radiotherapy	64 (51%)	178 (48%)
Resection		
Complete	44 (35%)	104 (28%)
Partial	52 (41%)	123 (33%)
Biopsy	30 (24%)	145 (39%)
Missing	0	1
MGMT promoter		
Methylated	42 (35%)	73 (35%)
Unmethylated	77 (65%)	136 (65%)
Missing / Inconclusive	7	164

Table 2: *IDH* mutated / G-CIMP positive patients. Note that patients 37 and 110 died without previous progression, while patient 125 had no progression until end of follow-up.

Patient ID	Histology	Treatment	*MGMT*	EFS (days)	OS (days)
37	AA	RT	M	196	196
110	GB	TMZ	M	582	582
125	GB	RT	M	924	924

GB, glioblastoma; AA, anaplastic astrocytoma; TMZ, temozolomide; RT, radiotherapy; M, methylated

Table 3: Baseline characteristics of the TCGA collective

	Patients < 65 years of age (n = 110)	Patients ≥ 65 years of age (n = 42)	
Mean age, years (range)	51,6 (23 – 64)	71,9 (65 – 83)	p < 0.0001
Median Karnofsky performance score (range)	80 (40 – 100)	80 (40 – 100)	p=0.0737
Extent of operation			p=1.0
Resection	97 (88%)	37 (88%)	
Biopsy	13 (12%)	5 (12%)	
Initial treatment			p=0.364
RT + TMZ	91 (83%)	32 (65%)	
RT alone	19 (17%)	10 (24%)	
MGMT promoter			p=0.851
Methylated	39 (35%)	16 (38%)	
Unmethylated	71 (65%)	26 (62%)	